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(54) Title: PREPARATION OF FROZEN FERMENTED FOODS USING ANTIFREEZE POLYPEPTIDE-EXPRESSING MICROORGANISMS

(57) Abstract

Methods and compositions for preparation of frozen fermented food products using antifreeze polypeptide-expressing microorganisms are provided. In particular the invention provides for use of fish antifreeze polypeptide-expressing microorganisms in fermentation of milk to produce and store frozen yogurt.

PREPARATION OF FROZEN FERMENTED FOODS USING ANTIFREEZE POLYPEPTIDE-EXPRESSING MICROORGANISMS

FIELD OF THE INVENTION

The present invention relates generally to methods and reagents useful in maintaining the quality of frozen food products during frozen storage, particularly enhanced storage life and the maintenance of consumer accepted quality of dairy products.

BACKGROUND OF THE INVENTION

Refrigeration, particularly freezing, is a common and preferred means for storing biological materials. Frozen storage generally arrests or considerably slows the deterioration of the biological product.

Frozen or refrigerated foods are now a mainstay of the human diet in developed nations. Thus extensive research has and is being carried out by food scientists to ensure high quality products for the consumers. This is particularly true with regard to frozen vegetables and frozen deserts such as ice cream and yogurt.

Frozen deserts such as ice cream or yogurt are generally eaten in the frozen state. Thus, the texture of the frozen product as well as its flavor is important to consumers. Texture is to a large extent governed by the size of the ice crystals. Producers of these frozen deserts have gone to considerable effort and expense to ensure smooth textured products. However, during frozen storage the ice crystals can grow and thus roughen and spoil this texture. The growth of ice crystals during frozen storage is known as recrystallization. This problem is particularly common when the frozen storage conditions are less than ideal, such as during transportation or storage in modern frost-free home freezers. After a relatively short period of time at above-zero temperatures (*i.e.*, above 0°C), or even at sustained freezing temperatures, frozen foods can become less desirable or even unsuitable for human consumption due to the ice recrystallization process.

Although manufacturers use a variety of techniques to reduce the damage associated with recrystallization success has been limited and significant problems

In a most preferred embodiment the invention comprises incubating milk with bacterial species *Lactobacillus balgaricus* and *Streptococcus lactis* that are capable of fermenting milk to produce yogurt and capable of secreting an ocean pout type III antifreeze polypeptide; incubating the bacteria and milk under conditions that produce yogurt; and freezing the yogurt at a temperature below -5°C, so as to produce frozen yogurt.

The invention also provides a composition comprising yogurt and a microorganism wherein the microorganism comprises a gene encoding a fish antifreeze polypeptide.

DETAILED DESCRIPTION

Definitions

As used herein, "fermentation" refers to the chemical conversion of carbohydrates or proteins in foods through the use of microorganisms. In this process carbohydrates are often converted to lactic acid.

As used herein, "food product" refers to a foodstuff (a substance that can be used, or prepared for use, as food) that can be transformed by the action of a fermenting microorganism to a fermented food product.

As used herein "fermented food product" refers to an edible food prepared by a process that includes fermentation by a microorganism.

As used herein "yogurt" refers to a dairy product produced by the lactic acid fermentation of milk by the action of microorganisms.

As used herein "Antifreeze polypeptides" (AFPs) refers to macromolecules found in the body fluids of some animals and plants, which have the commonly known property that they reduce non-colligatively the freezing point of water. Antifreeze polypeptides are also known as "thermal hysteresis proteins." As used herein, "antifreeze polypeptides" includes glycoproteins as well as chemically synthesized, and recombinantly produced polypeptides having a protein sequence with substantial similarity to a naturally occurring AFP and retaining the properties of an antifreeze polypeptide.

As used herein "fish antifreeze polypeptide" refers to an AFP that is found in nature in a fish, as well as chemically synthesized and recombinantly produced

polypeptides having a protein sequence with substantial similarity to a naturally occurring fish AFP and retaining the properties of a antifreeze polypeptides.

As used herein, "recombinantly produced polypeptides" refers to a polypeptide produced using recombinant DNA techniques. Recombinant DNA techniques are well known and are characterized by the joining of at least two segments of DNA that are not naturally joined in nature (*e.g.*, a bacterial promoter and a fish polypeptide coding sequence).

As used herein, "substantial similarity" denotes a characteristic of a polypeptide sequence or nucleic acid sequence, wherein the polypeptide sequence has at least 70 percent sequence identity, preferably 80 percent sequence identity, and most preferably 90% sequence identity compared to a reference sequence (*e.g.*, a naturally occurring antifreeze polypeptide), and the nucleic acid sequence has at least 80 percent sequence identity and preferably 90% sequence identity compared to a reference sequence. The reference sequence may be shorter than the full-length naturally occurring polypeptide or nucleic acid sequence but will be at least 12 residues long for the case of a polypeptide and at least 36 bases long for the case of a nucleic acid.

Description

The present invention provides methods for preparing a frozen fermented food product by adding a microorganism that is capable of fermenting the food product to produce the fermented food product and also is able to secrete a fish antifreeze polypeptide. The use of a microorganism that both secretes an AFP and ferments the food product has several advantages over other methods for affecting ice crystal formation and freezing temperature. For example, the claimed method avoids the costly necessity for purifying an AFP prior to addition to a food product. In addition, this will eliminate any possible contamination from the purification protocol and the pyrogenicity associated with foreign microorganisms. Furthermore, because the AFP is secreted by the fermenting microorganism of the claimed invention, this process requires fewer steps than other methods.

The food product of the invention is usually milk but other foods that are fermented to produce an edible fermented food may also be used. Examples include cabbage (which can be fermented to produce sauerkraut), cucumbers (which can be

Most preferred is microorganism capable of expressing an ocean pout type III antifreeze polypeptide. The ocean pout type III antifreeze polypeptide is preferred because it has no amino acid bias and has been shown to be active when expressed in *E. coli* (Li *et al.*, 1991, *Protein Engineering* 4:995-1012; Sönnichsen *et al.*, 1993, *Science*, 259:1154-1157). In addition, the type III AFP is preferred because type I AFP of winter flounder may not be stable at the fermentation temperature and type II AFP may not be correctly folded in bacterial system and is very susceptible to reduction.

The methods for engineering bacteria and fungi capable of expressing and secreting a heterologous polypeptide are well established (*see, e.g.*, Maniatis *et al.* (1982), *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York; Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 (Academic Press, Inc., San Diego, CA); Simon *et al.*, 1986, *Appl. Environ. Microbiol.* 52:394-395; and von Wright *et al.*, *1985*, *Appl. Environ. Microbiol.* 50:1100-1102, all of which are incorporated herein by reference)

The production of microorganisms capable of expressing and secreting an AFP can be carried out in a variety of ways that will be apparent to one of ordinary skill. The DNA sequence encoding the AFP will preferably be operably linked (*i.e.*, positioned to ensure the functioning of) to an operon which allows the DNA to be transcribed (into an RNA transcript) and translated into a polypeptide in the microorganism. Promoters for both bacteria and fungi are well known in the art. Preferred operons for expression in lactic acid bacteria include the lactose operon of *S. thermophilus* or lac ABCDFEGX operon of *L. lactic* because they have been used successfully to drive foreign gene expression in the hosts (*see, e.g.*, Simons *et al.*, 1993, *J. Bact.* 175:5186-5175; Mollet *et al.*, *1993*, *J. Bact.* 175:4315-4324).

The AFP may be expressed as a fusion polypeptide for increased stability or other beneficial properties. Furthermore the AFP polypeptide may be modified via a modification of the gene encoding the polypeptide. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (*see, e.g.*, Gillman and Smith, 1979, *Gene* 8:81-97 and Roberts *et al.*, 1987, *Nature* 328:731-734).

The microorganisms of the invention are capable of secreting the AFP. Accordingly, the AFP will preferably be linked to a signal peptide sequence. Examples of suitable signal peptide sequences include those from the usp45 gene of *L. lactis ssp*

Table 1

Names used to describe types of yogurt

Product Name	Country of Origin
Jugurt/Eyran/Ayran	Turkey, etc.
Busa	Turkestan
Kissel Mleka	Balkans
Urgotnic	Balkan Mountains
Leban/Laban	Lebanon/Arab countries
Zabady (Zabbady)	Egypt/Sudan
Mast/Dough	Iran/Afghanistan
Roba	Iraq
Dahi/Dadhi/Dahee	India
Mazun/Matzoon/Matsun/ Matsoni	Armenia
Katyk	Transcaucasia
Tiaourti	Greece
Cieddu	Italy
Mezzoradu	Sicily
Gioddu	Sardinia
Biokys	Czechoslovakia
Karmdinka	Poland
Tarho	Hungary
Tykmaelk/Ymer	Hungary
Villi (Fiili)	Finland
Filmjolk/Fillbunke/ Surmelk/Taettemjolk/ Tettemelk	Scandinavia
Iogurte	Brazil/Portugal
Proghurt	Chile
Skyr	Iceland
Gruzovina	Yugoslavia
Kefir/Donskaya/Varentes Kurunga/Koumiss/ Ryazhenka/Guslyanka	Soviet Union
Tarag	Mongolia
Shosim/Sho/Thara	Nepal

EXAMPLES

The invention is illustrated by the following examples. These examples are offered by way of illustration, not by way of limitation.

Example 1

Construction of a strain of lactic acid bacteria that produce antifreeze polypeptide - Method I

To engineer a lactic acid bacterium that produce antifreeze proteins several steps are involved. The first step is selection and preparation of a chromosomal site for the AFP gene integration. A native operon of a strain of lactic acid bacteria such as the lactose operon of the *S. thermophilus* or *L. lactis* genome, consisting of the *lacS* (lactose permease) and *lacZ* (β-galactosidase) genes, is used for the integration of an antifreeze protein gene. Integration of an AFP gene into such an operon should preserve its correct function. The AFP gene should become a functional part of the operon and be regulated similarly (*see*, *e.g.*, Simons *et al.*, 1993, *J. Bact.*, 175:5186-5175, and Mollet *et al.*, 1993, *J. Bact.* 175:4315-4324).

To do this, the *lacS* and *lacZ* genes from the host bacteria are cloned by PCR procedures or conventional gene cloning methods. At least one restriction enzyme site is generated between the two genes by designing particular primer sequences for the PCR reactions. The restriction enzyme sites are generated for convenient segment linkage and insertion of DNA fragment. An antibiotic resistance marker gene such as an ampicillin or erythromycin resistance gene is inserted into the generated restriction enzyme site. The lacS-Amp^R-lacZ DNA in an appropriate vector such as pNZ932 is transformed into the lactic acid bacterial strain (*see*, e.g. Simons *et al.*, 1993, *J. Bact.* 175:5186-5175). Ampicillin-resistant transformants will be selected, and gene integration will be verified using PCR and DNA sequencing.

The second step involves construction of an antifreeze protein gene cassette. Using the nucleotide sequence derived from a cloned ocean pout type III AFP gene, an appropriate type III AFP gene is assembled from synthetic oligonucleotides using the preferential codons of the host (*see*, e.g., Mercenier, 1990, *FEMS Microbiology Reviews*, 87:61-78, and van Asseldonk *et al.*, *1992*, *FEMS Microbiology Reviews* 88:73-92). To make a bacteria secrete AFP, a signal peptide sequence (SP) from homologous genes such as the usp45 gene of *L. lactis ssp lactis* MG 1363 and the

bacteria will not be damaged (45-48°C). The starter bacteria are applied to inoculate with pretreated homogenized milk at 30-45°C. When the acidity reaches a certain prescribed level such as 0.8% and the amount of AFP in the product reaches required concentration (1-100 mg/liter milk), the fermented milk is cooled to 15-20°C to suppress bacterial activity. These products are used to make soft frozen yogurt, hard frozen yogurt, and mousse yogurt by adding different percentages of fruit syrup, sugar, stabilizers, fruit juice and emulsifiers to a cold fermented milk base.

Example 4

Production of frozen yogurt

Soft frozen yogurt is made by adding 20% fruit syrup and stabilizers and emulsifiers to 80% of a cold fermented milk base, and then filling into containers with a 50-60% overrun using a normal ice cream freezer. The product is stored at 0-6°C.

Hard frozen yogurt is made of 35% fruit juice, the overrun is 70-80%, and storage temperature is below -25°C. Mousse yogurt is made by mixing a fermented milk base with a warm mousse base (a homogenized mixture of skim milk, sugar, stabilizers and emulsifiers). The product is stored at below 0°C.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

8. The method of claim 1 wherein the microorganism is a fungus.

9. The method of claim 8 wherein the fungus is a yeast.

10. The method of claim 9 wherein the yeast is selected from the group comprising *Torulopsis holmil*, *Saccharomyces fragilis*, *Saccharomyces cerevisiae*, *Saccharomyces lactis*, and *Candida pseudotropicalis*.

11. The method of claim 1 wherein the fish antifreeze polypeptide is from an ocean pout.

12. The method of claim 11 wherein the antifreeze polypeptide is a ocean pout type III antifreeze polypeptide.

13. A method for preparing frozen yogurt comprising the steps:

a) contacting milk with a microorganism capable of secreting a fish antifreeze polypeptide, wherein the microorganism is capable of fermenting milk to produce yogurt;

b) incubating the microorganism and milk under conditions that produce yogurt;

c) freezing the yogurt at a temperature below -5° C. so as to produce frozen yogurt.

14. The method of claim 13 wherein the microorganism is selected from the group: *Streptococcus cremoris*, *Streptococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citrovorum*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus jugurti*, *Lactobacillus lactis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Torulopsis holmil*, *Saccharomyces fragilis*, *Saccharomyces cerevisiae*, *Saccharomyces lactis*, and *Candida pseudotropicalis*.

15. The method of claim 13 wherein the antifreeze polypeptide is a ocean pout type III antifreeze polypeptide.

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCI/CA 95/00568

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A23L1/03 A23C9/123 A23C19/032

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A23L A23C A23G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 03617 (UNILEVER) 17 February 1994 see claims 1-13	1-17
A	PATENT ABSTRACTS OF JAPAN vol. 16 no. 13 (C-901) & JP,A,03 232896 (SUMITOMO CHEM CO) 16 October 1991, see abstract	1
A	EP,A,0 424 771 (SOCIÉTÉ DES PRODUITS NESTLÉ) 2 May 1991 see claims 1-22	1-17
A	WO,A,90 13571 (DNA PLANT TECHNOLOGY CO) 15 November 1990 see page 21, line 3 - line 17; claims 1-12	1-27

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inv ·onal Application No
PCI/CA 95/00568

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